Association Between Erythropoietin Gene Polymorphisms and Diabetic Retinopathy

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**Objective:** To determine whether sequence variation in the erythropoietin gene (EPO) is associated with the development of diabetic retinopathy (DR).

**Methods:** This was a multicenter study based on 518 subjects with long-standing diabetes mellitus (DM), 173 with type 1 DM (T1DM) and 345 with type 2 DM (T2DM). Study groups consisted of 233 control subjects with no DR, 155 subjects with nonproliferative DR, 126 with proliferative DR, and 90 with clinically significant macular edema. Subjects with end-stage renal disease were excluded. DNA extracted from blood of each subject was genotyped for 3 EPO single-nucleotide polymorphisms (SNPs).

**Results:** All 3 SNPs in EPO were associated with overall DR status in the combined T1DM and T2DM and T2DM alone groups (CC genotype of rs551238, \( P < .008 \); GG genotype of rs1617640, \( P < .008 \); and CC genotype of rs551238, \( P < .008 \)) in the multivariate analysis. The GCC haplotype was also associated with overall DR status in the combined DM and T2DM alone groups \( (P = .008) \) by multivariate analysis. All SNPs and the GCC haplotype were also associated with proliferative DR and clinically significant macular edema in the combined DM and T2DM alone groups. No associations were found with T1DM alone.

**Conclusion:** Sequence variation in EPO is associated with the risk of DR independent of duration of DM, degree of glycemic control, and nephropathy.

**Clinical Relevance:** Identifying EPO genetic markers for high risk of developing DR could lead to the possibility of developing novel treatments or preventive therapies.


**Diabetic Retinopathy (DR)** is an ocular microvascular complication of diabetes mellitus (DM). Up to 40% of individuals with DM will develop DR at some stage of their illness, with approximately 8% becoming sight threatening. The mechanisms underlying DR are still incompletely understood. Current models of DR suggest that damage to retinal blood vessels by long-standing hyperglycemia leads to retinal hypoxia, which stimulates the DNA-binding activity of hypoxia-inducible factor. Hypoxia-inducible factor upregulates a large number of hypoxia-inducible genes, including vascular endothelial growth factor and erythropoietin (EPO). Consequent increased expression of these cytokines may then contribute to DR progression.

EPO is a glycoprotein that plays a major role in stimulation of bone marrow stem cells and erythropoiesis. It has also been shown to stimulate proliferation, migration, and angiogenesis in vascular endothelial cells exposed to hypoxia. EPO messenger RNA is expressed in the human retina. Expression of EPO receptors in vascular endothelial cells and the retina has also been demonstrated. Many studies have reported a higher concentration of EPO in the vitreous of patients with DM and proliferative DR (PDR) when compared with controls. Animal studies have shown increased EPO concentrations in ischemic retinas and EPO inhibitors preventing neovascularization, further supporting the role of EPO in the development of PDR.

EPO expression is influenced by single-nucleotide polymorphisms (SNPs) in EPO. Interestingly, there is no correlation between the vitreous and plasma levels of EPO, suggesting increased vitreous EPO is due to increased local production.
of EPO in the retina\textsuperscript{14} rather than due to increased systemic EPO production by the kidney. The human EPO gene is located on chromosome 7q21.\textsuperscript{15} Recently, Tong et al\textsuperscript{12} genotyped 613 subjects with type 2 DM (T2DM) (374 with PDR and end-stage renal disease [ESRD] and 239 controls with complication-free DM) for 19 SNPs in 11 genes involved in angiogenesis, including EPO. The only significant association with DR was found at SNP rs1617640 in the promoter of EPO, where the T allele was significantly associated with PDR and ESRD (P = .00191). This finding was replicated in 2 type 1 DM (T1DM) cohorts (1244 with PDR with or without ESRD and 715 controls with complication-free DM) with all subjects being European American (overall \( P = 2.76 \times 10^{-11} \)). The TTA haplotype of SNPs rs1617640, rs507392, and rs551238 were also disease associated (\( P = .0005 \)).\textsuperscript{12} We aimed to determine whether the same EPO sequence variation was associated with DR development in Australian subjects with DM and without ESRD.

Subjects were recruited from Ophthalmology and Endocrine clinics of 3 tertiary hospitals in metropolitan Adelaide, South Australia. All 3 hospitals are within 15 km of each other and drawn from very similar if not identical populations. Ethics approval was obtained from the human research ethics committees of each hospital and informed consent was obtained from all participants. In total, 518 subjects with long-standing DM were recruited. This cohort consisted of 173 subjects with T1DM and 345 with T2DM. Subjects were excluded if they had ESRD, defined as stage V chronic kidney disease undergoing dialysis or renal transplantation. Ninety-three percent were white and of European descent, with the remainder of Asian and Middle Eastern descent. To test for any population stratification, allele frequencies between white and nonwhite individuals were compared. Because no difference was detected (\( \chi^2 = .43; P > .81 \)), all individuals were analyzed together. All participants were older than 18 years and required to have either T1DM or T2DM of at least 5 years’ duration and to be taking oral hypoglycemic medication or undergoing insulin therapy for DM.

Retinopathy status was obtained from the treating ophthalmologist and graded according to the Early Treatment Diabetic Retinopathy Study criteria.\textsuperscript{5} Blinding DR was defined as severe nonproliferative DR, PDR, or clinically significant macular edema (CSME). A detailed questionnaire containing information regarding ethnicity, age at diagnosis, family DM history, coexisting risk factors, systemic complications of DM, ocular complications as a result of DR, ocular history, smoking history, and alcohol intake was conducted. Blood pressure and body mass index were measured. Renal function tests (serum creatinine and urine albumin levels and albumin:creatinine ratio) and blood cholesterol and hemoglobin \( A_1c \) (Hb\textsubscript{A1c}) levels were obtained. Plasma glucose and lipid levels were obtained at the time of recruitment. Hypercholesterolemia was defined as a total cholesterol level greater than 212 mg/dL, (to convert to millimoles per liter, multiply by 0.0259) or current use of lipid-lowering medication. Nephropathy was defined as the presence of microalbuminuria (albumin level, 0.03-0.3 g per day) or macroalbuminuria (albumin level, \( >0.3 \) g per day) but not meeting ESRD criteria.

DNA was extracted from peripheral blood samples using the QIAamp Blood Maxi Kit (Qiagen, Valencia, California). The same 3 EPO SNPs reported in Tong et al 12 (rs1617640, rs507392, and rs551238) were genotyped in all individuals using the Sequenom iPLEX Gold chemistry (Sequenom, San Diego, California) on an Autoflex mass spectrometer (Sequenom, Brisbane, Australia) at the Australia Genome Research Facility, Brisbane. Three individuals for each genotype at each SNP were sequenced to confirm the integrity of the genotyping assay. Single-nucleotide polymorphism genotyping was checked for compliance with Hardy-Weinberg equilibrium (HWE) using \( \chi^2 \) test. Linkage disequilibrium between markers and allelic association tests were calculated in Haplovie 4.0 (http://www.broadinstitute.org/mpg/haplovie). Genotypic associations were assessed in SNPStats.\textsuperscript{17} Dominant and recessive models were considered with respect to the minor allele. Odds ratios were calculated in SPSS (version 15.0; SPSS Inc, Chicago, Illinois). Haplotypic associations were undertaken in Haplo Stats (version 1.2.1)\textsuperscript{13} in 1 block of linkage disequilibrium.

### METHODS

Of the 518 subjects genotyped, 233 subjects (67 T1DM and 166 T2DM) had no DR; 122 (50 T1DM and 72 T2DM), nonproliferative DR; 126 (46 T1DM and 80 T2DM), PDR; and 90 (24 T1DM and 66 T2DM) had CSME. Of the participants with DR, the grading for the worst affected eye was used, with a hierarchy PDR considered worse than nonproliferative DR. Clinically significant macular edema was also considered as an independent analysis. Some individuals fell into more than 1 group because CSME can co-occur with any of the other DR gradings. If either eye had CSME irrespective of other DR gradings, the patient was classified as having CSME. Subjects with T1DM and no DR had a significantly lower age, shorter disease duration, lower cholesterol level, and less hypertension when compared with subjects with T1DM and DR. Subjects with T2DM and no DR were more likely to be female and have shorter disease duration, lower Hb\textsubscript{A1c} level, and higher BMI when compared with subjects with T2DM and DR (Table 1).

All 3 SNPs were in strong linkage disequilibrium (\( D^2 = 1.0, r^2 = 1.0 \) for all comparisons) and were in HWE when assessed in the total cohort (\( P > .05 \)). When broken down by type of DM and DR status, participants with T2DM and no DR deviated from HWE (\( P = .009 \)) for all 3 SNPs. Genotype frequencies of all SNPs in both DM subgroups are shown in Table 2. Given the strong linkage disequilibrium observed, all 3 SNPs gave identical association results. All 3 SNPs in EPO were associated with DR status in the combined DM group (T1DM and T2DM) and T2DM alone. The GG genotype of rs1617640, CC genotype of rs507392, and CC genotype of rs551238 were all significantly associated with the presence of DR after adjustment for age, sex, Hb\textsubscript{A1c} level, duration of disease, and nephropathy in the combined DM group (\( P = .008 \), data not shown). In the subanalysis for type of DM, these genotypes were significantly associated with DR in the T2DM group (\( P = .006 \)) (Table 3) but not the
T1DM group, although the total number of subjects with T1DM was fewer, reducing the statistical power to detect an association in this group. In the multivariate analyses, further subclassification for the type of DR found all 3 SNPs to be associated with PDR, CSME, and blinding DR in the combined DM group (\(P = .030, P = .040,\) and \(P = .033,\) respectively) and T2DM alone (\(P = .020, P = .018,\) and \(P = .016,\) respectively). In addition to controlling for age, sex, HbA1c level, duration of disease, and nephropathy, further multivariate analyses were undertaken, also controlling for hypertension, hypercholesterolemia, BMI, and smoking. All 3 SNPs continued to be significantly associated with overall DR, PDR, and blinding DR in the combined DM group and T2DM alone (\(P < .05,\) data not shown). No associations were found in an allelic association model for any SNP and DR (or its subtypes).

### Table 1. Clinical Characteristics of Participants With No DR Compared With DR Cases in T1DM and T2DM

<table>
<thead>
<tr>
<th>Clinical Characteristic</th>
<th>T1DM</th>
<th>T2DM</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No DR (n=67)</td>
<td>DR (n=106)</td>
</tr>
<tr>
<td>Female, No. (%)</td>
<td>32 (48)</td>
<td>52 (49)</td>
</tr>
<tr>
<td>Age, y, mean (SD)</td>
<td>36.3 (14.6)</td>
<td>48.6 (16.0)</td>
</tr>
<tr>
<td>Disease duration, y, mean (SD)</td>
<td>12.43 (11.7)</td>
<td>28.1 (11.1)</td>
</tr>
<tr>
<td>HbA1c level, %, mean (SD)</td>
<td>7.5 (2.0)</td>
<td>9.2 (7.5)</td>
</tr>
<tr>
<td>BMI, mean (SD)</td>
<td>25.9 (6.7)</td>
<td>25.4 (10.1)</td>
</tr>
<tr>
<td>Hypercholesterolemia, No. (%)</td>
<td>22 (33)</td>
<td>51 (48)</td>
</tr>
<tr>
<td>Nephropathy, No. (%)</td>
<td>11 (16)</td>
<td>30 (28)</td>
</tr>
<tr>
<td>Smoker, No. (%)</td>
<td>34 (51)</td>
<td>34 (51)</td>
</tr>
<tr>
<td>Hypertension, No. (%)</td>
<td>23 (34)</td>
<td>34 (34)</td>
</tr>
</tbody>
</table>

### Table 2. Genotype Frequencies for Each SNP in Participants With No DR and DR Cases by Type of Diabetes Mellitus

<table>
<thead>
<tr>
<th>SNP</th>
<th>T1DM</th>
<th>T2DM</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No DR (n=67)</td>
<td>DR (n=106)</td>
</tr>
<tr>
<td>rs1617640</td>
<td>TT</td>
<td>24 (37)</td>
</tr>
<tr>
<td></td>
<td>TG</td>
<td>30 (46)</td>
</tr>
<tr>
<td></td>
<td>GG</td>
<td>11 (17)</td>
</tr>
<tr>
<td>rs507392</td>
<td>TT</td>
<td>24 (37)</td>
</tr>
<tr>
<td></td>
<td>TC</td>
<td>30 (46)</td>
</tr>
<tr>
<td></td>
<td>CC</td>
<td>11 (17)</td>
</tr>
<tr>
<td>rs551238</td>
<td>AA</td>
<td>24 (37)</td>
</tr>
<tr>
<td></td>
<td>AC</td>
<td>30 (46)</td>
</tr>
<tr>
<td></td>
<td>CC</td>
<td>11 (17)</td>
</tr>
</tbody>
</table>

### Table 3. P Values for Association of EPO SNPs With DR in T1DM and T2DM

<table>
<thead>
<tr>
<th>SNP</th>
<th>Minor allele</th>
<th>T1DM Unadjusted P Value</th>
<th>T1DM Adjusted P Value</th>
<th>T2DM Unadjusted P Value</th>
<th>T2DM Adjusted P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Dominant</td>
<td>Recessive</td>
<td>Dominant</td>
<td>Recessive</td>
</tr>
<tr>
<td>rs1617640</td>
<td>G</td>
<td>.770</td>
<td>.900</td>
<td>.760</td>
<td>.130</td>
</tr>
<tr>
<td>rs507392</td>
<td>C</td>
<td>.770</td>
<td>.900</td>
<td>.760</td>
<td>.130</td>
</tr>
<tr>
<td>rs551238</td>
<td>C</td>
<td>.770</td>
<td>.900</td>
<td>.760</td>
<td>.130</td>
</tr>
</tbody>
</table>

**Abbreviations:** DR, diabetic retinopathy; HbA1c, hemoglobin A1c; T1DM, type 1 diabetes mellitus; T2DM, type 2 diabetes mellitus.

**SI conversion factor:** To convert HbA1c to proportion of total hemoglobin, multiply by 0.01.
the combined DM group or T1DM or T2DM alone (P > .05, data not shown).

The GCC haplotype was found at a higher frequency in the combined DM and T2DM alone groups with DR than the control group. This association was significant under a recessive model after adjusting for sex, HbA1c level, duration of disease, and nephropathy (P = .008) (Table 4). This haplotype was also associated with PDR, CSME, and blinding DR in the combined DM group (P = .030, P = .040, and P = .008, respectively) and T2DM alone (P = .027, P = .031, and P = .009, respectively). No significant association was observed for T1DM alone.

**COMMENT**

EPO is an important cytokine that stimulates proliferation, migration, and angiogenesis in vascular endothelial cells. EPO expression has been shown to be influenced by SNPs in EPO and is elevated in the vitreous of subjects with PDR. EPO is thus a biologically plausible candidate gene with potential to influence susceptibility to develop DR and this study has investigated the association of EPO gene variation with DR development.

This study found an association between 3 EPO SNPs and DR in a cohort of subjects with DM, with the GCC haplotype having an increased frequency in patients with T2DM with DR. In contrast to our study, Tong et al identified the T allele of rs1617640 to be the risk-associated allele with PDR. It was also reported that the same allele in the EPO promoter region had a major effect on EPO messenger RNA transcription levels in an in vitro model. The opposite haplotype (TAA) was reported as the risk allele for PDR in their study. Similar overall allele frequencies (across combined cases and controls) were observed in the 2 studies. However, a major and important difference between the 2 cohorts is the complete lack of ESRD in the current study, compared with the majority of cases having ESRD in the Tong et al study. As opposed to Tong et al, this study did not find an association of the EPO SNPs with DR in T1DM. The substantially larger T1DM cohort of Tong et al had additional power, which may explain the lack of association observed in the current study.

End-stage renal disease leads to anemia through reduced EPO production. Although similar vascular processes may be involved in the pathogenesis of both diabetic nephropathy and DR, it is possible that different genetic factors play a role in their susceptibility. The development of DR and nephropathy have each been shown to have a strong genetic component. However, the majority of the chromosomes believed to be involved in the inheritance of nephropathy and retinopathy susceptibility are not shared. It is therefore possible that different variations within EPO play a role in DR and ESRD development and therefore possible that Tong et al have identified variations in EPO responsible only for ESRD, accounting for the differences between our and their findings. Previous studies have also suggested no association of plasma EPO levels with increased vitreous EPO, but rather increased vitreous levels to be due to local production of EPO in the retina. Further studies investigating factors influencing local and systemic production of EPO and tissue-specific effects of EPO gene regulation are also required to further understand the role of EPO in DR and ESRD pathogenesis.

This study is not the first to report an opposite allele/genotype of the same SNP to be associated with a disease. For example, the C allele of SNP rs3741916 of the glyceraldehyde-3-phosphate dehydrogenase gene have been reported by different studies to be significantly associated with Alzheimer disease in white subjects. This "flip flop" phenomenon may occur when a single locus association is found in the presence of multilocus effects and this single locus association may be confounded by other loci. Another reason can include sampling variation among the studies, whereby the magnitude of an association between an allele and risk of disease varies across different (especially ethnic) populations because of the presence of different linkage disequilibrium patterns. Finally, associations of opposite alleles of the same SNP may occur because of differences in its relationship with other causal variants, including environmental factors.

Another important consideration is the deviation from HWE observed in the T2DM no DR group. This group was as highly selected as the controls, and thus, the deviation is not unexpected in the presence of a true association. However, there is a chance that this deviation is due to population stratification. The T1DM groups conformed to HWE as did the T2DM with DR group, indicating that recruitment bias and genotyping errors are likely not the cause. However, the reported association does depend on the group that does not conform to HWE.

Subjects with no DR in our study had shorter duration of DM and fewer associated vasculopathic risk factors when compared with those with DR. We accept this as a limitation of our study; however, an attempt to over-

| Table 4. Association of Haplotypes With Blinding DR in T1DM and T2DMa |
|---------------------------|---------------------------|---------------------------|
| Alleles | T1DM | T2DM | T1DM | T2DM | T1DM | T2DM | T1DM | T2DM | T1DM | T2DM | T1DM | T2DM |
| Haplotype | Frequency | No DR Frequency | DR Frequency | P Value | Frequency | No DR Frequency | DR Frequency | P Value |
| 1 | T | T | A | 0.602 | 0.600 | 0.604 | .758 | 0.638 | 0.664 | 0.613 | .652 |
| 2 | G | C | C | 0.398 | 0.400 | 0.396 | .103 | 0.362 | 0.336 | 0.367 | .098 |

Abbreviations: DR, diabetic retinopathy; T1DM, type 1 diabetes mellitus; T2DM, type 2 diabetes mellitus.

aData for all haplotypes with a frequency of more than 2% in 2 linkage disequilibrium blocks are given. Single-nucleotide polymorphisms are numbered as in Table 2.
come these influences on the outcome of results has been made by adjusting for these factors in the multivariate analyses.

In conclusion, our results show that in an Australian white population, variation in EPO predicts the risk of developing DR, independent of duration of DM. There is clearly a need for further independent association studies to further explore the role of EPO sequence variation in DR susceptibility. Also, further functional characterization is required to better elucidate the role of EPO in DR and ESRD development. If confirmed, this finding leads to the possibility of developing treatments or preventive therapies for DR based on a clearer understanding of the mechanism of involvement of EPO in DR development.

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Author Contributions: Assoc Prof Craig had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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